Microcomputer-Automated Reactor for Synthesis of ^{13}C-Labeled Monosaccharides

For less labor-intensive, larger-scale, and more efficient production of D-[1-^{13}C] glucose and other labeled monosaccharides using the cyanohydrin reduction reaction, an automated reactor was designed, constructed, and tested. A suitable vessel and all ancillary and control equipment were fabricated, or otherwise obtained, and the apparatus was controlled with a microcomputer program written in BASIC. Running without manual intervention, the yields for the condensation reaction of D-arabinose with KCN and the recovery of the excess cyanide were high (>90%), and the resultant aldononitriles were reduced to product aldoses with little reduction of the intermediate imines to amines. The reduction rate could be improved by controlling pH between 2-4 and by agitation speeds >250 rpm, while no improvement resulted from the use of high hydrogen pressures [>129 kPa (4 psig)]. Reduction reactions conducted in the presence of formic acid (to regulate pH) suggest that formic acid acted as a coreducing agent.

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Introduction

Stable, isotopically-enriched compounds play an increasingly important role in the study of biological metabolism and the diagnosis and treatment of human disease (cf. Bottomley, 1989; Rabbenstein et al., 1988). This growth has been stimulated, in large part, by dramatic technical developments in biological nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). For example, image-selected *in-vivo* spectroscopy (ISIS) (Ordidge et al., 1986), and magnetic resonance imaging (MRI) (Mansfield and Morris, 1982) are descendants of the basic NMR method that provide new means to probe the nature of biological processes. Clinical MRI is confined at the present time to the observation of protons, but the observation of other isotopes (e.g., ^{13}C) is likely to become important in the future. The incorporation of stable isotopes (e.g., ^{2}H, ^{15}N, ^{13}C), either uniformly or site-specifically, into biomacromolecules such as proteins and nucleic acids has assisted greatly in their structural characterization by NMR methods by simplifying otherwise complex spectra (cf., Westler et al., 1988; LeMaster and Richards, 1988). These stable isotope-aided NMR methods are expected to have a profound influence on the fields of chemistry, biology and clinical medicine. For these reasons, the development and refinement of methods to introduce stable isotopes into biomolecules remains an important and contemporary problem.

D-Glucose plays a central role in biological metabolism, being the preferred carbon source for most cells. D-[1-^{13}C] glucose has been used in numerous studies of biological metabolism (including a recent *in vivo* study comparing human muscle glycogen synthesis in diabetics and normal subjects, Shulman et al., 1990), because this labeled monosaccharide can be prepared by a reliable, high-yield cyanohydrin reduction method (Figure 1) (Serianni et al., 1979, 1987) that is readily adapted to small scale batch synthesis (Serianni, 1988). D-arabinose is condensed with a threefold excess of [^{13}C]-labeled hydrogen cyanide at pH 7.0–7.5 to produce C-2 epimeric D-[1-^{13}C] aldononitriles. After recovering the excess cyanide, the nitriles are reduced *in situ* with a heterogeneous palladium catalyst at pH 4.3 to generate an epimeric mixture of imines, which hydrolyze rapidly to give D-[1-^{13}C] mannose and D-[1-^{13}C] glucose. The reduction step is performed under acidic conditions to maximize imine hydrolysis and minimize imine reduction to amines. The [1-^{13}C] aldohexoses are separated by chromatography on Dowex 50 × 8 (200–400 mesh) ion-exchange resin in the calcium form (Angyal et al., 1979) and crystallized from anhydrous methanol.

The anticipated increase in demand for labeled sugars in the future provided the impetus to evaluate the feasibility of constructing and operating an automated synthesizer. This

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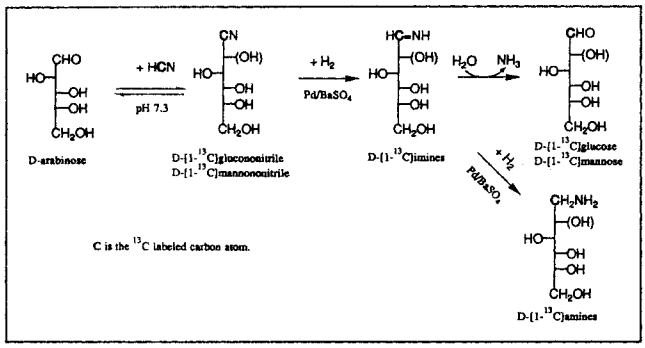


Figure 1. Reaction scheme for the synthesis of D-[1-^{13}C] glucose by the cyanohydrin reduction method.

study describes the construction of a reaction vessel, in which all of the synthesis steps up to and including the reduction step are performed under computer control; the final product purification by chromatography was not automated and has been described elsewhere (Serianni, 1988). The equipment assembled for the automated control of this reaction vessel, including the microcomputer, I/O board, relay control board, and various sensors, is briefly described in this paper. Some testing and refinement of the device have also been performed and the results are reported here. In the latter regard, the apparatus was used to study the effects of several reaction parameters, especially the reduction conditions, on the reaction rate using unlabeled cyanide. The results show that pH and agitation rate are critical parameters for the reduction reaction, while the effect of hydrogen pressure was negligible.

Experimental Studies

The apparatus

A detailed description of the apparatus is given elsewhere (Stafford, 1990), and only the fundamental features are described here. The apparatus was assembled as an integral unit on a stand (Figure 2). The reaction vessel, which was rounded to withstand high reduction pressures, was constructed in two halves of borosilicate glass, with the bottom jacketed half supported by an adjustable jack and the top half secured with a metal band clamp. The two halves of the vessel were sealed with a neoprene O-ring secured with acrylic flanges.

All liquid reagents were held in borosilicate glass reservoirs that were positioned above the reaction vessel. The solid catalyst was prepared as an aqueous slurry and was contained in a reservoir constructed in two parts and sealed with an O-ring and clamp. Plastic solenoid valves (ASCO) were used to control all reagent additions (gases and liquids), with the exception of acid and base which were added using stainless steel control valves (ASCO). Check valves were used on the nitrogen, cyanide trap, and vacuum lines to prevent backflow. Reagent gases originated in cylinders equipped with Matheson regulators (one stage for hydrogen; two stage for nitrogen), and vacuum was drawn with a water aspirator or house vacuum line. Two traps equipped with gas diffusers were used in series to trap excess cyanide (Serianni and Barker, 1987). A pressure sensor was connected to the vessel with Tygon tubing.

Solution pH was measured with a deep vessel combination electrode modified to fit in the vessel and to be compatible with the operation pressures. An overhead stirrer with a special

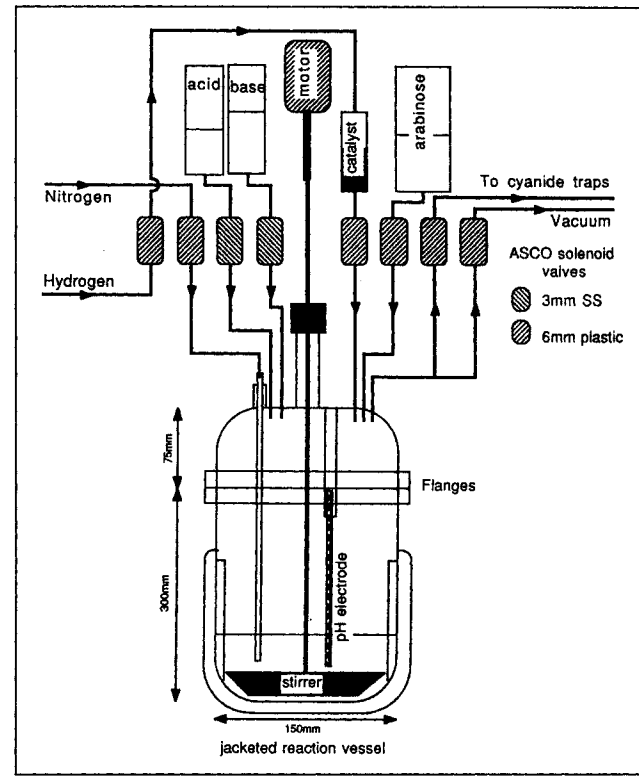


Figure 2. The apparatus.

leakfree assembly (Ace) was used to provide mixing during each step of the reaction. The stirrer shaft (60 mm × 10 mm), with a modified Teflon stirring blade, was rotated by an electric motor, which was secured at the top of the stand with a laboratory clamp.

The control equipment and computer program

The apparatus was controlled by a computer, input/output interfaces, and relays. An IBM PC-AT-compatible microcomputer system with MS-DOS was used to control the automation, and the control program was written in GW-BASIC. A DASCON-1 (Metrabyte) I/O board, was used to control the apparatus. A high impedance interface, "pH by PC" (Computer Chemistry Inc.), was used to interface the pH electrode, the RTD temperature measurement probe, the pressure sensor, and 12 digital I/O channels from the DASCON-1.

A relay control board (Stafford, 1990) served as an interface between the 5 VDC output signals of the I/O board, via the "pH by PC" unit, and the 110 VAC mains power that was used to operate the control valves and agitator. Reed relays were used to operate the control valves, and 3A relays were used to control the stirring motor. The pressure transducer, which was connected to the reaction vessel with Tygon tubing, was also mounted on this board.

The operation of the automated reactor was controlled with a structured computer program (Figure 3) written in GW-BASIC. The program consists of subroutines selected from the main menu to perform various tasks that include testing and preparing the apparatus, running the reaction, and reviewing data accumulated during reaction runs. These functions, especially the reaction run routine, are explained in more detail in

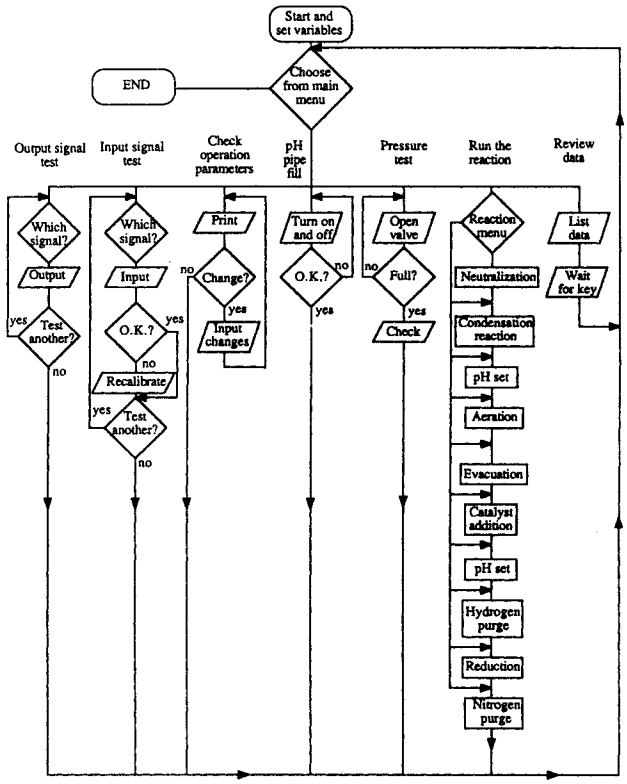


Figure 3. Flowchart of the control program.

the next section while full details of their operations are available elsewhere (Stafford, 1990).

Experimental procedure

The reagents and reaction conditions, including reagent quantities, used in the experimental procedure are listed in Tables 1 and 2, respectively.

The computer and relay control board were turned on, and the control program was automatically loaded and run. The pH electrode was then checked and recalibrated if necessary using standard buffers at pH 4.0 and 7.0. An aqueous solution of D-arabinose was transferred to the sugar reservoir on the apparatus. The solutions for the cyanide traps were prepared by dissolving KOH in water and methanol *in situ*, and the traps

Table 1. Reagents Used for the Synthesis of D-Glucose

Reagents	Manufacturer/Distributor
D-Arabinose	Sigma Chemical Co.
Palladium (5%) on barium sulfate	Sigma Chemical Co.
Potassium cyanide (99.2%)	Fisher Scientific
Potassium hydroxide (87.8%)	Fisher Scientific
Acetic acid (99.7%)	Fisher Scientific
Hydrogen, industrial-grade (99.95%)	Linde
Nitrogen, industrial-grade (99.97%)	Linde
Methanol, industrial-grade	Warsaw Chemical
Distilled water	In-house reagent

Table 2. Default Reaction Conditions

Quantity of D-arabinose	260 mmol disolved in 175 mL of water
Quantity of potassium cyanide	780 mmol disolved in 500 mL of water
Quantity of glacial acetic acid (acid)	300 mL
Quantity of 1 M KOH solution (base)	50 mL
Condensation reaction duration	20 min
Condensation reaction pH	7.3
Condensation reaction temp.	20–25°C
Aeration duration	8 h
Aeration pH	4.5
Nitrogen pressure during aeration	125 kPa (3.5 psig)
Contents of trap #1	750 mL methanol, 50 mL water, 38.6 g KOH
Contents of trap #2	290 mL methanol, 10 mL water, 19.3 g KOH
Evacuation duration	5 min
Initial reduction pH	4.3
Quantity of catalyst	23 g of used and 2 g of new
Agitator speed	240 rpm
Hydrogen pres. (lower & upper limit)	136–170 kPa (5—10 psig)
Vol. of mixture during reduction	1 L

were then sealed. The $Pd/BaSO_4$ catalyst was prepared as a slurry with water and transferred into its reservoir, which was filled with water and sealed; to facilitate reliable comparisons of different reaction parameters (e.g., reduction pH), catalyst charges were identical for the series under consideration. Glacial acetic acid and 1 M KOH were added to the acid and base reservoirs. Finally, KCN and water were added to the reaction vessel, which was then sealed. The overhead stirrer was activated to check for proper alignment and to dissolve the cyanide.

The nitrogen regulator was opened [set manually at 125 kPa (3.5 psig)] to pressure-test the reaction vessel. The parameters for the reaction (e.g., pH, reduction pressure) were examined and modified from the default values if desired. The vacuum and hydrogen lines (the latter regulator was set at the higher reduction pressure) were turned on, and the acid and base flows were tested.

After completing all of these manual reactor setups and checks, the automated reaction sequence was initiated from the computer keyboard, after entering a name for the reaction record file. The computer then proceeded to perform all the reaction steps, aside from turning the cooling water on and off, without manual intervention. The cooling water was turned on during cyanide neutralization and off once the pH had been set properly for the cyanide condensation reaction (pH 7.3); this neutralization is the only step for which cooling is needed, whereas room temperature is preferred for the remaining stages. The computer proceeded with the neutralization by creating a slight vacuum (to ensure that acid is added) in the reaction vessel; acetic acid was added automatically until pH 7.3 was reached. The pressure was again lowered to aid in the addition of the D-arabinose solution. During the condensation reaction, the solution pH was maintained at 7.3 ($\pm$0.05) for 15–20 min, and slow stirring (~60 rpm) was employed. After completion of the condensation step, the solution pH was adjusted to 4.5, and the

mixture was aerated with nitrogen for ~8 h. The effluent was vented through two methanolic KOH traps to remove excess cyanide. Faster agitation (~240 rpm) was used during the aeration step to ensure optimal cyanide removal and recovery. After cyanide removal, the vessel was evacuated with stirring for 5–20 min to remove residual cyanide, which inhibits the reduction step, and the catalyst was added by opening the catalyst and hydrogen valves simultaneously. The pH was automatically adjusted to 4.3 with the addition of acetic acid, the vessel was evacuated and filled once to remove most gaseous impurities, and the reduction was initiated with fast agitation. Hydrogen pressure was maintained between two limiting values [typically 136-170 kPa (5–10 psig)] by refilling the vessel to the upper pressure limit when the lower limit was reached. The final reaction step was an evacuation followed by a nitrogen purge to remove hydrogen and allow safe opening of the reaction vessel.

During operation, the computer recorded the starting time of each step, the pressure, temperature, and pH at 5 minute intervals. These data were stored permanently in a data file and displayed temporarily on the computer screen. This record was useful in assessing the extent of reduction, and checking the operating parameters employed during each step.

When the reaction was complete, the reaction mixture was removed manually and vacuum filtered through a 7 cm glass fiber filter (Whatman GF/B) to remove the catalyst. The catalyst was recovered from the filter and stored under 10% acetic acid for reuse (aged catalyst). A natural abundance ^{1}H-decoupled ^{13}C NMR spectrum of the product mixture was obtained at 75 MHz on a General Electric GN-300 fourier-transform NMR spectrometer to detect and quantify the relative amounts of nitrile, aldose and amine. The quantity of cyanide recovered in the traps was determined by silver nitrate assay (Meites, 1963).

For reasons of cost, and to allow the detection and assay of arabinose relative to the product hexoses by natural abundance ^{13}C NMR, the experiments in this study were conducted with normal KCN (1.1% ^{13}C); the considerably more expensive K^{13}CN can be substituted with no change in the above protocol to prepare C-1 labeled sugars. Separation of the C-2 epimeric aldohexoses was not attempted, as this procedure has been described in detail previously (Serianni, 1988).

Results and Discussion

The reaction conditions used in previous small-scale manual batch synthesis of aldoses by the cyanohydrin reduction method (Serianni, 1988) provided a reasonable starting point for this study (Table 2). The effects of varying some of the experimental conditions on the fundamental parameters of the reaction (e.g., reaction rates and product yields) are discussed in the following sections.

Cyanide condensation

The initial reaction of the process is the reversible condensation of D-arabinose with hydrogen cyanide to form D-mannono- and gluconononitriles. A threefold excess of cyanide was used in this step to insure a high (>90%) conversion of D-arabinose to the epimeric nitriles (Serianni et al., 1979). While it may be argued that cyanide recovery could be effectively eliminated if D-arabinose was used in excess instead, the residual D-arabinose makes the chromatographic purification of the product hexoses more difficult, and thus this approach is considered less attractive. Under standard conditions, the reaction was conducted at room temperature for 15–20 min. Several syntheses were performed with some variations in these conditions (15–25°C, 15–30 min). However, the resultant ^{13}C NMR spectral analyses of the final reaction mixtures showed that the amount of unreacted D-arabinose (<10%) did not vary significantly with these conditions, and the *gluco:manno* ratio remained relatively constant at ~1:2.

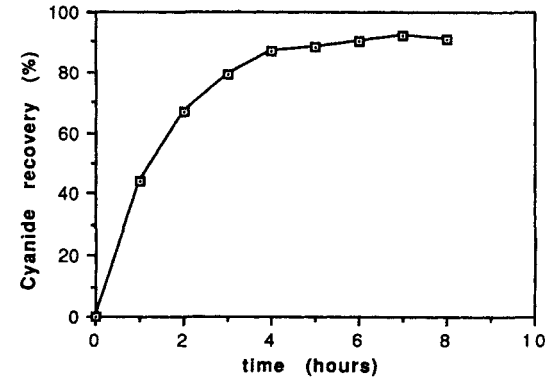


Figure 4. Cyanide recovery vs. time for a constant flow of nitrogen.

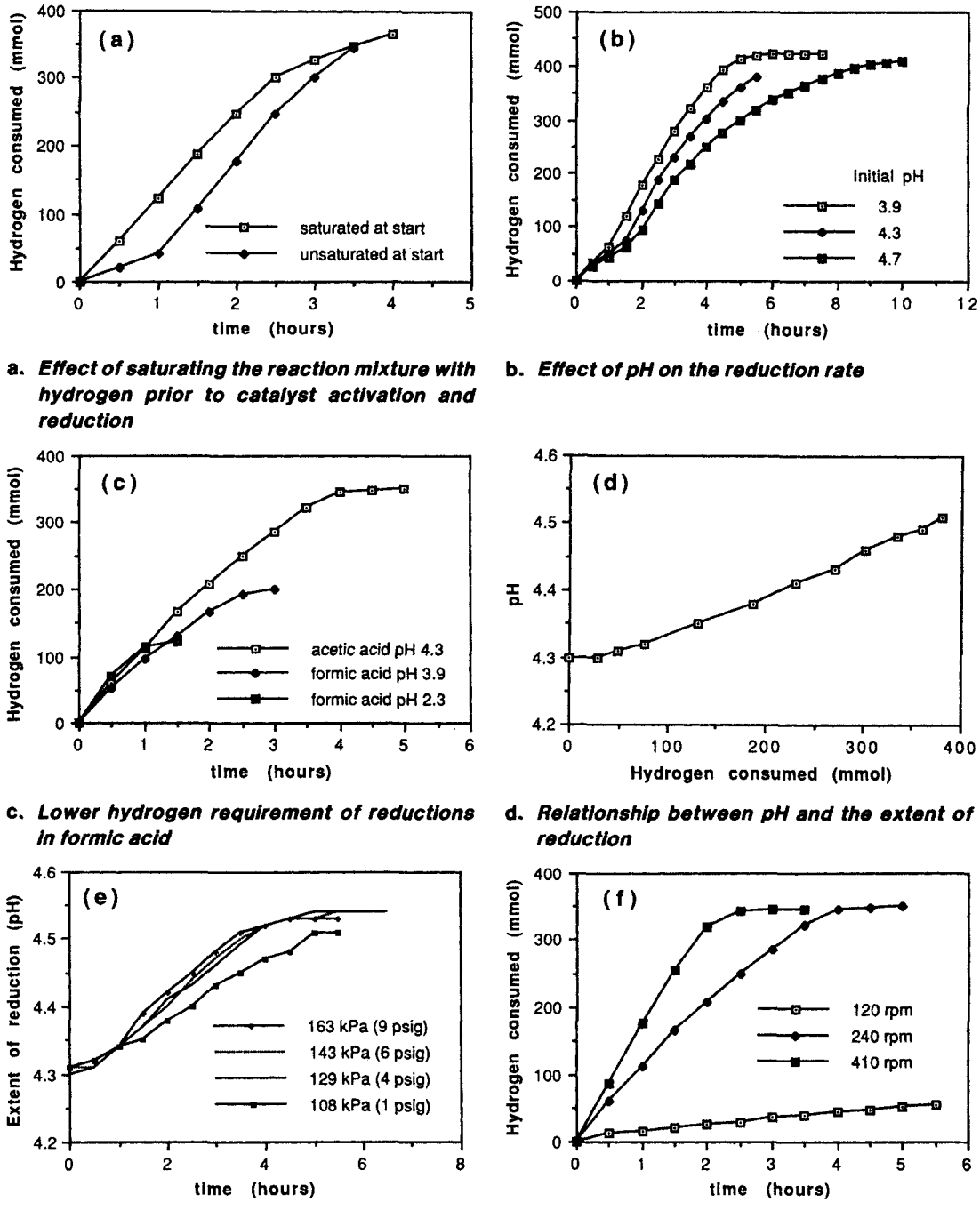
Cyanide recovery

The excess cyanide from the condensation reaction was recovered by aerating the reaction mixture with nitrogen and passing the HCN-laden gas through two methanolic KOH traps connected in series. Most of the excess cyanide (~90%) was recovered in the first trap after 5 hours and the passing of ~570 std. L of nitrogen at 125 kPa (3.5 psig). The recovery was only slightly enhanced with longer aeration times (Figure 4), although it might be improved by using a larger volume percent water in the trapping solutions, since cyanide is more soluble in water than in methanol (Merck Index, 1983).

Catalytic reduction

The final step of the process involves the conversion of aldononitriles to product aldoses (see Figure 1). This three-phase catalytic reduction was studied extensively, because several important variables can significantly affect the reaction rate, product selectivity and yield (Serianni et al., 1979; Serianni et al., 1980). The effects of pH, "catalyst age" (extent of previous use of the catalyst), hydrogen pressure, agitation speed, and batch size were investigated in this study, and the results are summarized in Figures 5a-5f. Although these results indicate a slight overconsumption of hydrogen as a consequence of overreduction and/or leakage, the relative trends are correctly represented.

The reduction reaction failed to occur if *all* of the catalyst added to the reaction mixture was not prereduced. Upon addition of some prereduced catalyst (usually catalyst used in a previous reduction), the reaction proceeded rapidly. This result may be caused by the formation of a complex between residual cyanide (or the nitriles) and the active sites on the unactivated palladium catalyst (Cotton and Wilkinson, 1988), which excludes hydrogen and inhibits catalyst reduction. However, in the presence of some prereduced catalyst with hydrogen bound to its active sites, the inhibiting species may be reduced (catalytic



a. ***Effect of saturating the reaction mixture with hydrogen prior to catalyst activation and reduction***

b. ***Effect of pH on the reduction rate***

c. ***Lower hydrogen requirement of reductions in formic acid***

d. ***Relationship between pH and the extent of reduction***

e. ***Effect of hydrogen pressure on the reduction rate***

f. ***Effect of agitator speed on the reduction rate***

Figure 5. Effects of operating variables during the reduction step.

reduction of cyanides and nitriles to amine and ammonia is well known, cf. Barrett and Titley, 1919; Tinapp, 1969), thereby liberating free catalyst sites that are required for nitrile reduction.

The catalyst used in previous reductions (aged catalyst) remained active in subsequent reductions, although the reaction rate was slower than when mixed with new catalyst. When the reaction was conducted with similar quantities of different batches of used catalyst, catalyst activity became a variable parameter. Consequently, studies of the effects of pressure and pH were made using identical catalyst mixtures.

The typical reduction curve contained three components comprising a general sigmoidal shape. The early flat component (lag time) was absent in reductions of reaction mixtures where hydrogen saturation was achieved prior to the addition of some activated catalyst (Figure 5a). Thus the lag time may reflect the time needed for the hydrogen to diffuse through the liquid and adsorb onto the catalyst surface. Alternatively, traces of resid-

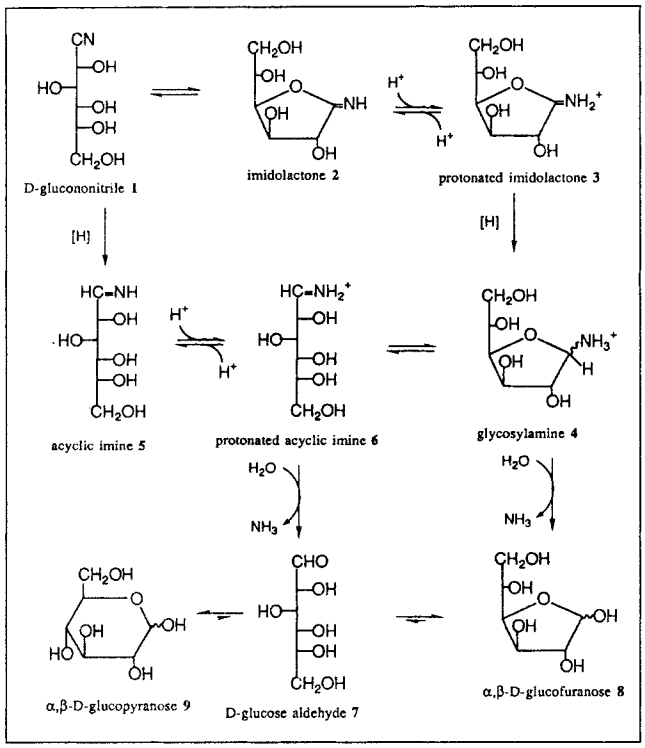


Figure 6. Postulated reaction scheme for D-gluconononitrile reduction and imine hydrolysis.

A similar scheme may be written for D-mannononitrile.

ual cyanide may bind strongly at the active sites (Cotton and Wilkinson, 1988), and the time required to slowly reduce this cyanide prior to the start of the nitrile reduction may account for the lag time.

The steep component of the curve was approximately linear, implying that the reduction rate was independent of nitrile concentration. Toward the end of the reduction, however, when nitrile concentration was low, this linearity was lost.

The rate of catalytic reduction depended on the acidity of the reaction mixture, with increasing $[H^+]$ stimulating the reaction rate (Figure 5b). This observation may be explained if the protonated imidolactone **3** (Figure 6) is the reactive form during reduction. After the reduction of **3** to **4**, the latter may hydrolyze to **8** either directly or indirectly through **6** and **7**. Compound **8** spontaneously equilibrates to **9** via **7**, with **9** being the predominant form of D-glucose in aqueous solution. The alternate route ($1 \rightarrow 5 \rightarrow 6 \rightarrow 7 \rightarrow 9$) is less likely, as the reduction kinetics should not be affected by solution pH in this case.

While there is an obvious benefit of lower pH in enhancing the reduction rate, the reaction yield is affected only slightly. At pH $\ll$ 4, there is a slight increase in the quantity of byproduct amines produced as expected (Tinapp, 1969), although this increase was not accurately quantified in this work. Poorer yields were also obtained at higher pH (>4.7) suggesting that a pH of ~4 is near optimal for this reaction.

Reductions were also performed at low pH (~2.3) by substituting formic acid for acetic acid, since the former has a pK value ~1 unit lower than that of acetic acid. Formic acid reductions (Figure 5c) proceeded more rapidly than those performed with acetic acid (with equivalent yields) and consumed less hydrogen than was required stoichiometrically. The lower consumption of H_2 and a rise in reactor pressure during and after the reaction was complete, suggested that formic acid acted as a partial reducing agent, liberating CO_2 in the process. The use of formic acid as a reducing agent of nitro-compounds with metal catalysts has been described previously (Entwhistle et al., 1977), and indeed, Shiue and Wolf (1985) used formic acid as a reducing agent to prepare D-[1-^{11}C] glucose in moderate yield by a modified version of the cyanohydrin reduction reaction using Raney alloy as a catalyst. The use of formic acid instead of hydrogen gas may be advantageous for the synthesis of D-[1-^{13}C] glucose if the yield could be improved. The use of potentially dangerous hydrogen gas could be eliminated from the protocol, and less vigorous agitation would be required for the two-phase reduction reaction. Attempts to reduce aldononitriles with formic acid and $Pd/BaSO_4$ in the absence of hydrogen were partially successful (~25% completion), but the use of formic acid to lower pH and facilitate more rapid reduction has immediate practical benefit.

The pH of the reaction mixture increased by ~0.24 pH units during the course of reductions in acetic acid, although this increase was smaller when lower initial pH values were chosen, due to the larger quantities of acetic acid that were added to lower the pH. The increase in pH was proportional to the uptake of hydrogen gas (Figure 5d) and stopped when the reduction was complete. Thus, this pH change is a useful parameter to monitor the state of the reduction reaction. The rise in pH is caused by the consumption of acid by the ammonia liberated during imine hydrolysis (Figure 6). However, during reductions in formic acid, the pH and pressure continued to rise after nitrile reduction was complete, presumably due to the catalytic degradation of formic acid to CO_2 and H_2.

Under otherwise identical conditions, no benefit was gained by using high hydrogen pressures during catalytic reduction; reduction curves obtained with H_2 pressures between 129–163 kPa (4–9 psig) were indistinguishable (Figure 5e). However, slower rates were observed when pressures near atmospheric pressure were employed. These results imply that a critical pressure between 108–129 kPa (1–4 psig) is needed to introduce hydrogen gas into the liquid phase at a rate commensurate with its consumption rate. Therefore, future reactors could be constructed for relatively low design pressures.

Solution agitation speed was found to critically affect the reduction rate. For the three-phase reduction reaction, good dispersion of hydrogen gas was required for the reaction to ensue (Figure 5f). The reaction barely proceeded at an agitation speed of 120 rpm. A standard speed of 240 rpm was sufficient for reduction, but faster reduction rates were observed at higher speeds (e.g., 410 rpm). The reaction rate was not as dependent on agitator speed as predicted by Perry et al., 1984 (exponent 1.7 vs. 2.5), but it appears to be more dependent on this parameter than on the mass transfer rate from liquid to solid. Nienow and Miles (1978) found for a two-blade flat paddle (0.75 times the diameter of the vessel with a small vessel bottom clearance) that the solid/liquid mass transfer coefficient depended on agitator speed with an exponent of 0.68. Levins and Glastonbury (1978), while finding a similar figure of 0.62, also identify other correlations whose exponents vary from 0 to 1.4. All of these exponents, however, are smaller than that measured for this system (1.7), implying that gas dispersion was important. Nevertheless, it was undesirable to run the reaction at higher speeds on a regular basis, for fear of damaging the

vessel or stirrer assembly. In future designs, it may be possible to utilize a more robust agitation system that could be reliably operated at higher, more efficient speeds.

The reactor was operated successfully with a larger batch size (520 mmol of sugars reduced in 1.5 L), but the reduction proceeded more slowly (~twice as long as 260 mmol). Volumes of ~1 L were reduced with ease, but there was a practical limit to the volume of the reaction mixture, above which mass transfer was ineffective in the present vessel. This slower reduction rate could be countered by using a higher stirring speed, by changing the vessel geometry to improve mixing, or simply by running the reaction for a longer time.

In practice, a potential problem with the reduction step is to determine the end point. Because of possible gas leakage, the amount of hydrogen consumed during the reaction may not always be a reliable guide. Similarly, time may also not be a satisfactory parameter because of the variability of catalyst activity, and the varying rates of reduction when different quantities of reagents are used. The pH rise during the reduction, a parameter not monitored in previous studies of this reaction, shows promise toward this end. There are some problems with this measurement when the vessel is being agitated, due to the formation of gas bubbles that causes fluctuations in the measured reading. This difficulty, however, may be overcome with appropriate computer software to filter these variations.

In summary, the automated synthesizer described in this study reliably conducted all components of the cyanohydrin reduction reaction with yields >90%, thus demonstrating that microcomputer-controlled automation of this method to synthesize isotopically labeled sugars is feasible.

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